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The UHRF1 Protein Stimulates the Activity and Specificity of the Maintenance DNA Methyltransferase DNMT1 by an Allosteric Mechanism*

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Background: UHRF1-mediated targeting of DNMT1 to replicated DNA is essential for DNA methylation.
Results: We show here that UHRF1 stimulates the activity and specificity of DNMT1 by an allosteric mechanism.
Conclusion: UHRF1 has multiple roles that support DNA methylation including targeting and regulation of the activity and specificity of DNMT1.
Significance: Regulation of DNMT1 is essential for the rapid and faithful remethylation of DNA after replication.

The ubiquitin-like, containing PHD and RING finger domains protein 1 (UHRF1) is essential for maintenance DNA methylation by DNA methyltransferase 1 (DNMT1). UHRF1 has been shown to recruit DNMT1 to replicated DNA by the ability of its SET and RING-associated (SRA) domain to bind to hemimethylated DNA. Here, we demonstrate that UHRF1 also increases the activity of DNMT1 by almost 5-fold. This stimulation is mediated by a direct interaction of both proteins through the SRA domain of UHRF1 and the replication focus targeting sequence domain of DNMT1, and it does not require DNA binding by the SRA domain. Disruption of the interaction between DNMT1 and UHRF1 by replacement of key residues in the replication focus targeting sequence domain led to a strong reduction of DNMT1 stimulation. Additionally, the interaction with UHRF1 increased the specificity of DNMT1 for methylation of hemimethylated CpG sites. These findings show that apart from the targeting of DNMT1 to the replicated DNA UHRF1 increases the activity and specificity of DNMT1, thus exerting a multifaceted influence on the maintenance of DNA methylation.

Epigenetic signals are defined as heritable, but flexible, marks that affect gene expression and other chromatin-related processes. They include DNA methylation and the modifications of histone proteins (1, 2). DNA methylation in mammals occurs at the 5-position of cytosines mainly within CpG sites, about 60–70% of which are methylated on average (3–5). Methylation of CpG-rich regions in gene promoters is a repressive epigenetic signal. DNA methylation is established during embryogenesis, development, and gametogenesis where cell type-specific methylation patterns are generated that later are maintained within cell lineages (6, 7). The methyltransferase DNMT1[3] recognizes and preferentially methylates hemimethylated CpG sites about 10–40-fold faster than unmethylated sites (8–16), which constitutes an important molecular mechanism for the inheritance of DNA methylation patterns during DNA replication and cell division. DNMT1 is a highly processive enzyme, making it suitable for methylation of long stretches of hemimethylated CpG sites formed after DNA replication (9, 10, 17, 18). Mouse DNMT1 comprises 1620 amino acid residues and contains several domains (see Fig. 1). The specificity of DNMT1 can be explained on the basis of the crystal structure of a DNMT1 C-terminal fragment containing bromo adjacent homology (BAH) 1, BAH2, and catalytic domains in complex with hemimethylated DNA (12, 14). Structures of larger DNMT1 fragments provided evidence for complex allosteric regulation of its activity and specificity. The *CXXC* domain has been implicated in an autoinhibitory function (11), but the role of the *CXXC* domain in the context of the full-length protein has not yet been clarified (13). Additionally, the crystal structure of DNMT1(291–1620) without DNA (19) showed that the replication focus targeting sequence (RFTS) domain occupied the DNA binding cleft in the catalytic domain of DNMT1, and the *CXXC* domain was moved aside. This finding suggested an autoinhibitory role of the RFTS domain that was supported by enzyme kinetics (19, 20).

Furthermore, DNMT1 interacts with several proteins, which may affect its activity and specificity. The interaction with the proliferative cell nuclear antigen, a component of DNA replication forks, facilitates loading of DNMT1 on newly synthesized DNA (21). In addition, DNMT1 interacts with the UHRF1 protein, which specifically binds hemimethylated CpG sites via its SET and RING-associated (SRA) domain (22–25). The mouse UHRF1 comprises 782 amino acid residues and contains several domains (Fig. 1). The SRA domain of UHRF1 and two regions of DNMT1 (the RFTS domain and amino acid residues

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[3] The abbreviations used are: DNMT, DNA methyltransferase; UHRF1, ubiquitin-like, containing PHD and RING finger domains protein 1; PHD, plant homeodomain; RING, really interesting new gene; SRA, SET and RING-associated; BAH, bromo adjacent homology; RFTS, replication focus targeting sequence; AdoMet, *S*-adenosylmethionine.

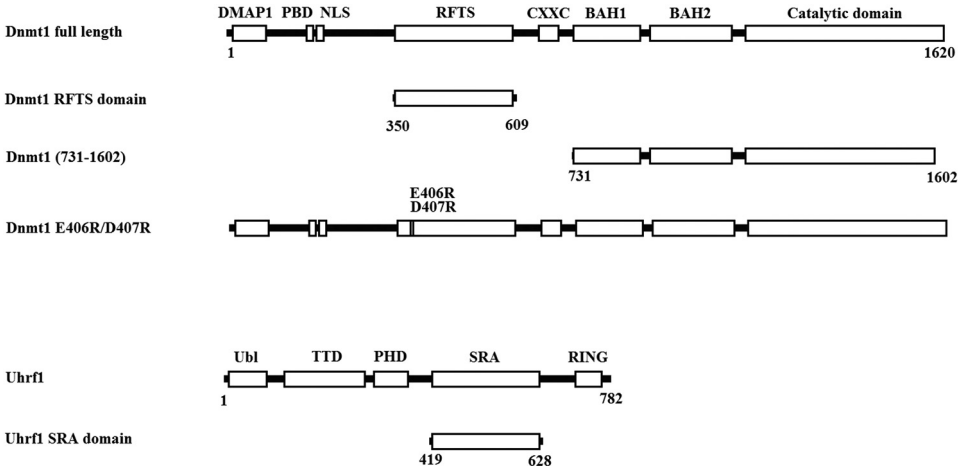

FIGURE 1. **Schematic representation of the domain architecture of DNMT1 and UHRF1 and the fragments used in this study.** DNMT1 comprises an N-terminal DMAP1-interacting domain, a proliferative cell nuclear antigen-interacting region (*PBD*), a nuclear localization sequence (*NLS*), a RFTS domain, a *CXX*C domain, two BAH domains, and a catalytic domain. Starting from the N terminus, the UHRF1 protein comprises a ubiquitin-like (*Ubl*) domain, a tandem tudor domain (*TTD*), a PHD, an SRA domain, and a RING domain.

1081–1408) were shown to be involved in the interaction between these proteins (22, 26). Cellular studies demonstrated that DNMT1 and UHRF1 co-localize within the nucleus during S phase, and both are enriched at sites of active DNA replication (26, 27), indicating that UHRF1 targets the methyltransferase to newly replicated hemimethylated DNA (23, 26). This process can enhance the specificity of DNA maintenance methylation because it increases the presence of DNMT1 on replicated DNA, whereas its presence is reduced on the parental DNA, which does not contain valid hemimethylated target sites (28).

In addition to the SRA domain, two more domains of UHRF1 are involved in its interaction with chromatin. The tandem tudor domain recognizes H3K9me3 in combination with unmethylated H3K4 (24, 29). Binding to H3K9me3 is important for the heterochromatic localization of UHRF1 and for the regulation of gene expression (29). Additionally, the interaction of UHRF1 with H3K9me3 is required for DNA methylation because a UHRF1 mutant defective in H3K9me3 binding cannot restore DNA methylation levels in UHRF1 knockdown cells (30). The plant homeodomain (PHD) of UHRF1 binds histone H3 tails unmodified at arginine 2 (H3R2) (31–33), and it was shown to be involved in the reorganization of the pericentromeric heterochromatin during replication of DNA (34). In fact, UHRF1 can bind H3R2 and H3K9me3 of one H3 tail simultaneously via the PHD and the tandem tudor domain, respectively (35–37), and the coordinated recognition of both histone marks is required for the maintenance of DNA methylation (38). In addition, the UHRF1 really interesting new gene (RING) domain possesses an E3 ubiquitin ligase activity, which was shown to ubiquitinate histones and DNMT1 and thereby regulate the chromatin structure and stability of DNMT1 (39–43).

Despite its well documented role as a maintenance methyltransferase, the internal preference of DNMT1 for hemimethylated CpG sites does not suffice to faithfully copy DNA methylation patterns. Moreover, the catalytic rates of DNMT1 observed by several groups are in the range of 0.1–1 turnover/min (8, 9, 11, 15, 44), which appears insufficient to explain the rapid kinetics of remethylation of CpG sites after DNA replication (45). These observations suggest that additional factors that increase the activity and specificity of DNMT1 exist. The DNMT1-proliferative cell nuclear antigen interaction is partially dispensable for the maintenance of DNA methylation because the deletion of the proliferative cell nuclear antigen binding domain in DNMT1 resulted only in a 2-fold delay of the remethylation (45–47). In contrast, the UHRF1 protein is absolutely essential to maintain cellular DNA methylation as illustrated by the phenotype of UHRF1 knock-out mice, which mimic DNMT1 knock-outs and show a severe loss of DNA methylation (26, 27). This finding may suggest that UHRF1 has additional roles in DNA methylation beyond the targeting of DNMT1. In this work, we investigated the effect of UHRF1 on the DNA methylation activity of DNMT1 by *in vitro* kinetics with purified proteins and show that UHRF1 stimulates DNMT1 by an allosteric interaction and that it increases its preference for methylation of hemimethylated DNA.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Mouse DNMT1 wild type (NCBI Reference Sequence NP_034196.5) and the DNMT1 E406R/D407R mutant were expressed using a baculovirus expression system according to the manufacturer's instructions (Bac-to-Bac manual, Invitrogen) and as described previously (13, 16). Both proteins were cloned into pFastBacHTa as N-terminal His_6-YFP fusions. The DNMT1 RFTS domain (amino acid residues 350–609) was cloned into pET28 (Novagen) as described (16), expressed in *Escherichia coli* BL21 (DE3, pLys) (Novagen). The DNMT1(731–1602) construct, a gift from Dr. D. Patel (Memorial Sloan-Kettering Cancer Center), was expressed in *E. coli* BL21 (DE3, pLys). UHRF1 full length (NCBI Reference Sequence NP_035061.3) was cloned from cDNA isolated from mouse embryonic fibroblast cells into pET28a as an N-terminal His_6 fusion (Novagen), and the UHRF1 SRA domain corresponding to amino acid residues 419–628 was cloned into pGEX-6P2 as an N-terminal GST fusion. The DNMT1 and the SRA domain variants were generated by site-directed mutagenesis as described (48). Full-length UHRF1 and the SRA domain variants were expressed in

E. coli BL21 (DE3, Codon+RIL) and *E. coli* BL21 cells (Novagen), respectively. All His-tagged proteins were purified with nickel-nitrilotriacetic acid-agarose (Genaxxon). GST-tagged proteins were purified with glutathione-Sepharose 4B (GE Healthcare) following standard procedures.

In Vitro DNA Methylation Assay—DNA methylation assays were performed as described previously (16). If not otherwise indicated, methylation reactions were carried out in the presence of 2 μM DNA substrate, 1.125 μM [methyl-^{3}H]AdoMet (PerkinElmer Life Sciences), and 0.2 μM recombinant DNMT1 at 37 °C in methylation buffer (20 mM HEPES (pH 7.5), 1 mM EDTA, 50 mM KCl, 0.1 mg/ml BSA). DNA substrates for the assay were prepared by the annealing of a 30-mer oligonucleotide (TTG CAC TCT CCT CC**CG** GAA GTC CCA GCT TC) containing one unmethylated CpG site (bold underlined) with an unmethylated or methylated complementary oligonucleotide to form unmethylated and hemimethylated substrates, respectively. In most experiments, DNMT1 was preincubated with UHRF1 or the SRA domain of UHRF1 (4 μM if not otherwise stated) for 15 min to form complexes before the methylation reactions were started by the addition of DNA. In control samples, DNMT1 was preincubated with the same volume of UHRF1/SRA dialysis buffer. Initial slopes were determined by linear regression. Methylation assays were calibrated by complete methylation of the substrates with M.SssI (New England Biolabs). The dependence of the reaction on the concentrations of DNA and AdoMet was determined by conducting experiments at different concentrations as indicated in the text. Data were fitted to a bimolecular binding equilibrium because under single turnover conditions the concentration dependence of the reaction rate depends on the saturation of the enzyme with substrate. The concentration dependence of the stimulation of DNMT1 by UHRF1 was determined in an analogous manner.

DNA Binding of SRA Domain Mutants—DNA binding of SRA domain mutants was investigated using an electrophoretic mobility shift assay essentially as described previously (13). Radioactively labeled hemimethylated 30-mer DNA was incubated with increasing concentrations of the SRA domain wild type and mutants (0.25, 0.5, and 1 μM) for 20 min at 22 °C. Afterward, mixtures were resolved on an 8% polyacrylamide gel, the resulting gel was dried, and the image was captured on x-ray film.

GST Pulldown Protein-Protein Interaction Assay—DNMT1 and GST-tagged SRA domain (or GST as control) were incubated in buffer (100 mM HEPES (pH 7.2), 1 mM EDTA, 25 μg/ml BSA, 0.5 mM DTT) at 37 °C for 20 min. Then they were incubated for 30 min at 4 °C with 20 μl of glutathione-Sepharose beads equilibrated in the same buffer. The beads were washed twice with the same buffer and centrifuged at 1000 × *g* for 1 min. After washing, the beads were resuspended in 25 μl of 2× SDS-PAGE sample loading buffer, heated to 95 °C for 10 min, and run on a 10% SDS-polyacrylamide gel. The proteins were transferred onto a PVDF membrane and incubated first with anti-GFP antibody (Clontech) at 1:2000 dilution and then with anti-rabbit HRP-conjugated antibody (Clontech) at 1:1000 dilution. After washing, the signal was developed with ECL reagent (GE Healthcare) and captured on x-ray film.

Alpha Screen Protein-Protein Interaction Assay—Interactions between DNMT1 variants and the SRA domain were measured using an Alpha Screen assay (PerkinElmer Life Sciences) according to the manufacturer's guidelines using an EnSpire multimode plate reader (PerkinElmer Life Sciences). Briefly, His-tagged DNMT1 wild type and variants (DNMT1 RFTS domain, DNMT1(731–1602), and DNMT1 E406R/D407R) were preincubated with the GST-tagged SRA domain or GST control in a half-volume 96-well plate (PerkinElmer Life Sciences) at room temperature for 1 h. Then glutathione donor beads and nickel chelate acceptor beads (PerkinElmer Life Sciences) were added to the solution, and the plates were incubated for 1 h at room temperature in the dark to allow for binding of tagged proteins to the corresponding beads. Interaction of both proteins brings the donor and acceptor beads in close proximity and allows excitation of acceptor beads by singlet oxygen produced after illumination of the donor beads. All proteins and beads were diluted in AlphaLISA universal buffer (PerkinElmer Life Sciences) containing PBS (pH 7.2) and 0.1% BSA. The final concentration of proteins in the assay was 0.25 μM, and the final concentration of both beads was 20 μg/ml.

Data Analysis—*p* values were determined by two-tailed *t* test using paired data of unstimulated and stimulated DNMT1 in Figs. 2*F* and 3*C* or by *t* test using -fold stimulation and preferences assuming an unequal variance in Figs. 4*C* and 5*B*.

RESULTS

The DNA Methylation Activity of DNMT1 Is Increased in the Presence of UHRF1—It was shown that UHRF1 recruits DNMT1 to hemimethylated DNA during DNA replication (26, 27). This interaction is crucial for the maintenance of DNA methylation patterns because the knock-out of UHRF1 resulted in a massive reduction of DNA methylation levels despite the presence of DNMT1 in the cells. Based on this observation, we speculated that UHRF1 could be involved not only in the targeting of DNMT1 but also in the regulation of its activity. To investigate a possible influence of UHRF1 on the activity of DNMT1, we conducted *in vitro* DNA methylation assays in the presence and absence of UHRF1. As a substrate, we used a 30-mer DNA with one centrally located hemimethylated CpG site. The substrate was incubated with recombinant full-length DNMT1 and AdoMet containing a radioactively labeled methyl group, and the progression of the reaction was followed by the incorporation of radioactivity into the DNA.

Initial experiments indicated that the rate of DNA methylation by DNMT1 under our conditions was 1.3 h^{-1} (Fig. 2*A*). Because the slow turnover did not allow us to conduct multiple turnover experiments, we followed the initial reaction phase under excess DNA and AdoMet and extracted the single turnover rate of the reaction, which is a well established approach to study enzyme mechanisms. Under these conditions, the rate of the reaction (v) is determined by the enzyme concentration (c_E), the single turnover rate constant (k_{st}), and the fractional saturation of the enzyme with substrates (f).

$$v = c_E \times k_{st} \times f \quad \text{(Eq. 1)}$$

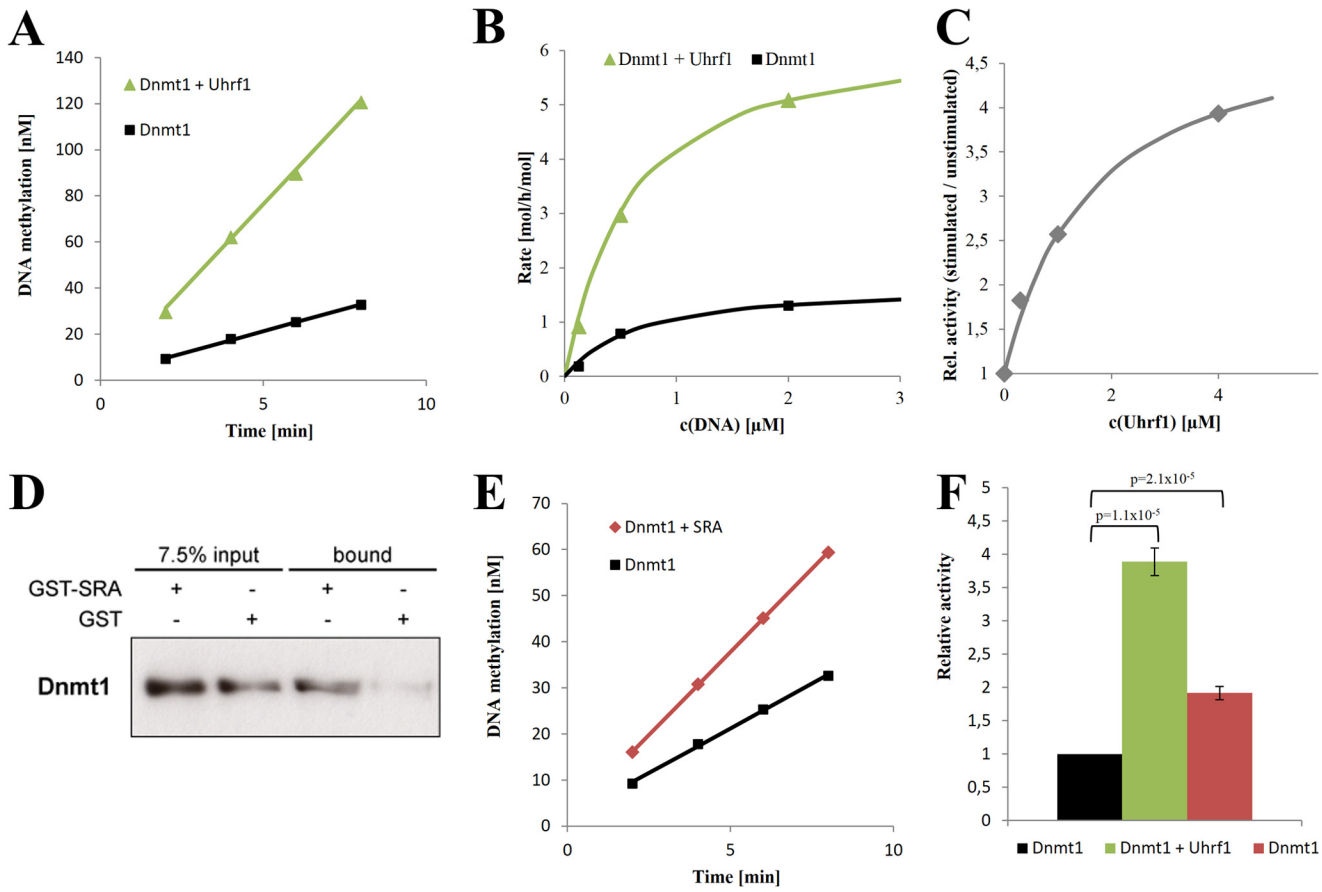

FIGURE 2. **Stimulation of DNA methylation activity of DNMT1 by UHRF1 or its SRA domain.** *A*, example kinetics of the methylation of hemimethylated DNA by DNMT1. Reactions were started by adding the DNA substrate and AdoMet after preincubation of DNMT1 with UHRF1 (*green triangles*) or after preincubation of DNMT1 with the same volume of UHRF1 dialysis buffer as a control (*black squares*). *B*, example of the dependence of the DNA methylation rate on the DNA concentration in the absence (*black*) and presence of UHRF1 (*green*). Data were fitted by a binding equilibrium yielding a K_d of 0.5 ± 0.1 μM in both cases (S.E. based on three experiments). *C*, example of the dependence of the stimulation of DNMT1 on the concentration of the UHRF1. The *solid line* shows a fit of the data to a binding equilibrium yielding a maximal stimulation of 4.7 ± 0.3-fold (S.E. based on three experiments). *D*, the UHRF1 SRA domain interacts with DNMT1. Purified GST-tagged SRA domain or GST alone was incubated with purified DNMT1, and complexes were pulled down with glutathione-Sepharose beads. The input and bound fractions were subjected to Western blotting and detected using anti-YFP antibody. The signal corresponding to DNMT1 is shown. *E*, example kinetics showing the stimulation of DNMT1 by the UHRF1 SRA domain (*red diamonds*) in comparison with preincubation with the same volume of dialysis buffer (*black squares*). *F*, compilation of the relative activity of DNMT1 in the absence and presence of UHRF1 and SRA (average of 10 and eight experiments for UHRF1 and SRA, respectively). *Error bars* represent SEM.

Experiments carried out at different concentrations of DNA showed an increase in the reaction rate with increasing DNA concentration, which reflects the increase in *f* due to a larger fraction of DNMT1 with bound DNA (Fig. 2*B*). Fitting of these data to a bimolecular binding equilibrium allowed us to determine that the K_d of DNA binding to DNMT1 under our conditions was 0.5 ± 0.1 μM. Similar experiments conducted at higher concentration of AdoMet yielded a K_d for AdoMet binding of 6.1 ± 0.3 μM. Based on these values, the single turnover rate constant of DNMT1 fully saturated with DNA and AdoMet can be extrapolated to about 11.5 ± 1 h^{-1}. These numbers are in good agreement with published data (for example, see Ref. 11).

Next, methylation experiments were conducted in the presence of full-length recombinant UHRF1. To allow for complex formation of UHRF1 and DNMT1, both proteins were preincubated together in the methylation buffer without DNA. In control reactions, DNMT1 was incubated with the same amount of UHRF1 dialysis buffer. After preincubation, the DNA methylation reactions were started by adding the DNA substrate and AdoMet. We observed that DNMT1 preincubated with UHRF1 was 3.9 times more active than DNMT1 incubated with UHRF1 buffer (Fig. 2, *A* and *F*), demonstrating that the interaction with UHRF1 leads to the activation of DNMT1. Nonspecific effects were excluded by adding BSA (0.1 mg/ml) to all reactions. Further controls showed that addition of more BSA (up to 0.3 mg/ml) did not change the outcome of these experiments. Methylation experiments of DNMT1 preincubated with UHRF1 were conducted at different DNA concentrations and showed that UHRF1 did not change the DNA binding affinity of DNMT1 (Fig. 2*B*). We investigated the dependence of the DNMT1 stimulation on the UHRF1 concentration (Fig. 2*C*), which allowed us to extrapolate that full saturation of DNMT1 with UHRF1 would lead to a 4.7-fold stimulation. Notably, the setup of this experiment did not allow UHRF1 to target DNMT1 to the substrate because the DNA substrate contained only one hemimethylated CpG site, indicating that a direct stimulation of DNMT1 must occur.

The DNA Methylation Activity of DNMT1 Is Also Increased in the Presence of the UHRF1 SRA Domain—We were interested to identify the domain(s) of UHRF1 that are responsible for the activation of DNMT1. It was shown previously that the

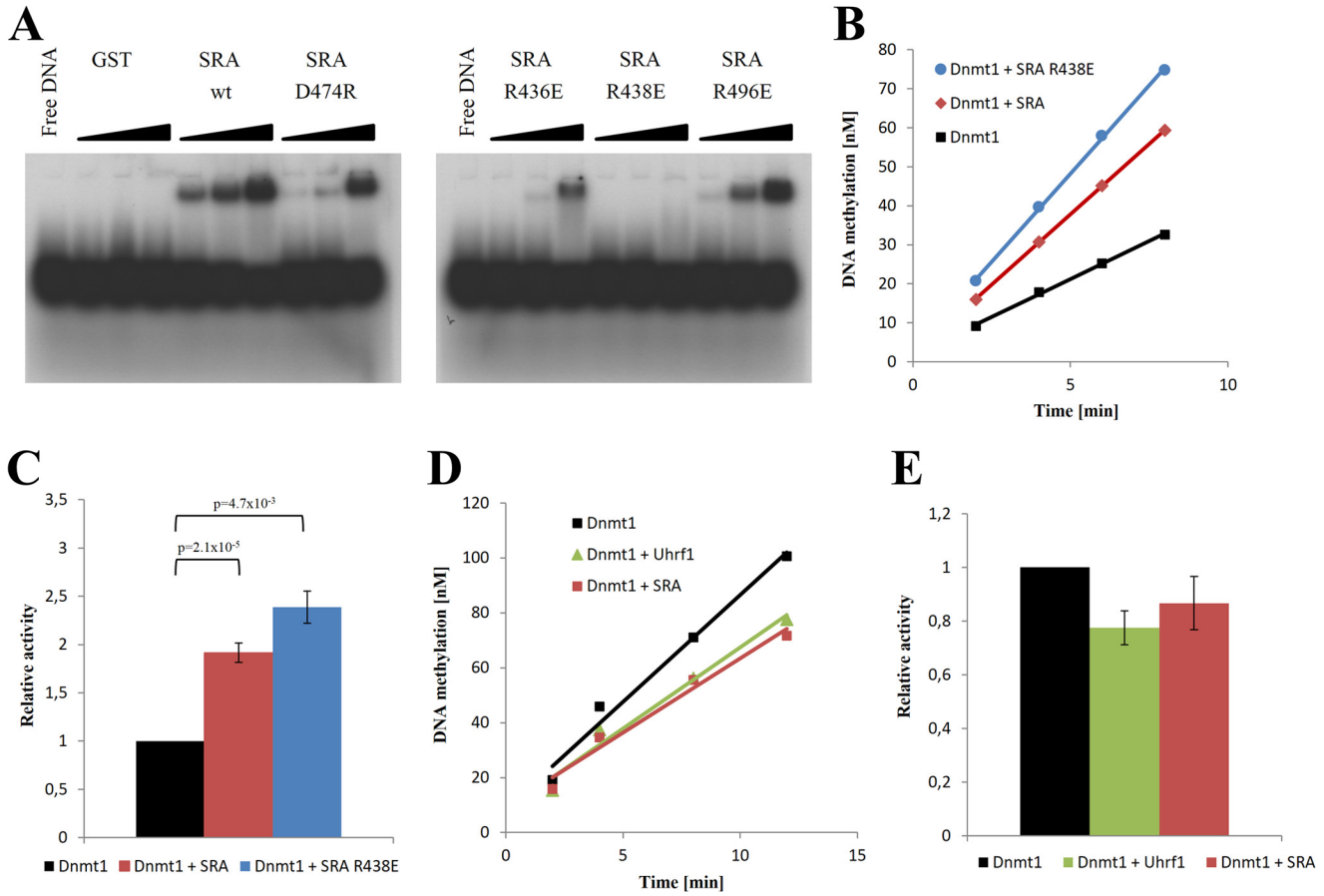

FIGURE 3. **The SRA domain of UHRF1 stimulates the activity of DNMT1 in a DNA binding-independent manner.** *A*, DNA binding of the GST-tagged SRA domain and its variants was analyzed by electrophoretic mobility shift assay. SRA variants (0.25, 0.5, and 1 μM) and GST control (1.25, 2.5, and 5 μM) were incubated with radioactively labeled hemimethylated DNA, and DNA binding was analyzed by gel retardation. SRA R438E demonstrated the strongest reduction of DNA binding. *B*, example kinetics of the methylation of hemimethylated DNA by DNMT1 preincubated with the SRA domain (*red diamonds*), SRA R438E mutant (*blue circles*), or dialysis buffer (*black squares*). *C*, summary of the activity of DNMT1 in the absence and presence of the SRA domain (data taken from Fig. 2*F*) or its R438E variant (average of three experiments). *D*, examples of DNA methylation kinetics. UHRF1 and SRA were preincubated with DNA, and the methylation reactions were started by adding DNMT1. *E*, absence of stimulation of DNMT1 if UHRF1 or SRA was preincubated with DNA (average of three experiments). *Error bars* represent SEM.

UHRF1 SRA domain that binds hemimethylated CpG sites also interacts with DNMT1 (22). We purified the isolated UHRF1 SRA domain and confirmed its interaction with DNMT1 in GST pulldown experiments (Fig. 2*D*). Next, we wanted to determine whether the isolated SRA domain also mediates an activation of DNMT1. As shown in Fig. 2, *E* and *F*, preincubation of DNMT1 with the SRA domain resulted in a 1.9-fold increase in activity of DNMT1, indicating that the isolated SRA domain is capable of stimulating DNMT1. The reduced level of stimulation as compared with full-length UHRF1 may be due to a weaker binding of the domain to DNMT1 when compared with full-length UHRF1.

DNA Binding of the SRA Domain Is Not Required for Stimulation of DNMT1—UHRF1 binds to hemimethylated CpG sites during DNA replication via its SRA domain. To examine whether DNA binding by the SRA domain was required for the stimulation of DNMT1, we generated SRA domain mutants, which lost DNA binding. According to the crystal structures of the SRA domain with bound DNA (23–25), the DNA binding interface of the SRA domain contains several arginine residues, which are engaged in electrostatic interactions with the DNA backbone. We selected the arginine residues 436, 438, and 496 as candidates for the mutagenesis. To achieve a maximum effect, we exchanged these arginines to glutamic acid. One additional mutant was generated in the 5-methylcytosine binding pocket of SRA where aspartic acid 474 was exchanged to arginine. All four mutants were successfully purified, and their DNA binding was investigated by electrophoretic mobility shift assays using the hemimethylated 30-mer DNA substrate (Fig. 3*A*). All mutants showed a reduction in DNA binding in comparison with wild type SRA. The strongest effect was observed with the R438E variant, which did not exhibit any detectable DNA binding in our experiments. Therefore, we decided to use the SRA R438E variant in the DNA methylation assay with DNMT1. Preincubation of DNMT1 with the SRA R438E mutant caused a 2.4-fold increase of DNMT1 activity, which is even slightly higher than the stimulation observed with the wild type SRA domain (Fig. 3, *B* and *C*). This result indicates that DNA binding of the SRA domain is not required for the stimulation of DNMT1. The slight increase in the stimulation observed with the SRA mutant as compared with the wild type SRA domain might be explained by the fact that the SRA mutant no longer bound to the DNA, and consequently, it was not competing with DNMT1 for the DNA substrate.

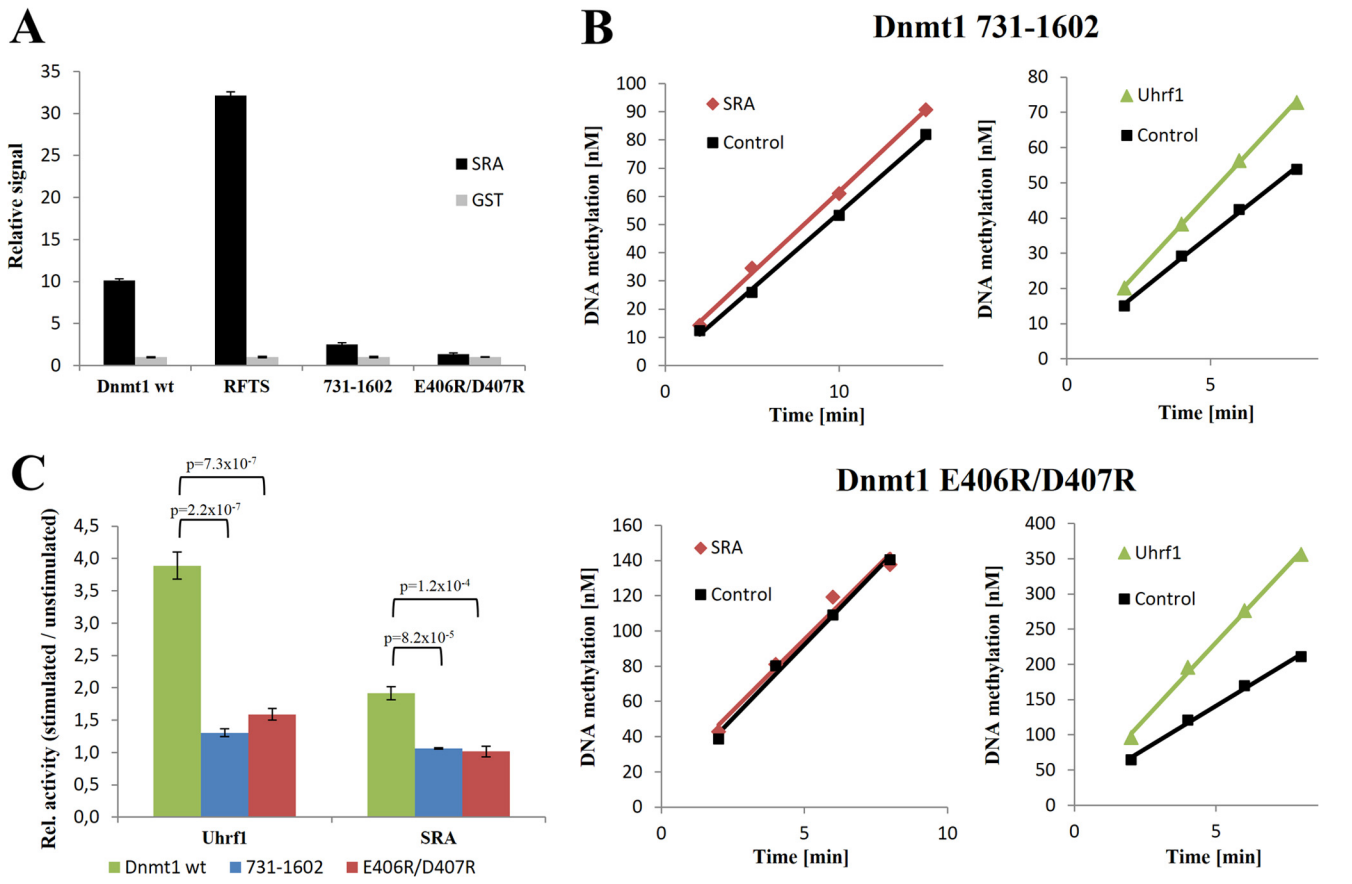

FIGURE 4. **UHRF1 stimulates the activity of DNMT1 via an interaction with the RFTS domain.** *A*, interaction of GST-SRA and His-tagged DNMT1 variants tested by Alpha Screen assays. The data are presented as Alpha signals relative to the background probed by incubation of DNMT1 or its variants with GST (average of three experiments). *B*, example kinetics of the methylation of hemimethylated DNA by the DNMT1 E406R/D407R variant and the truncated DNMT1(731–1602) in the absence (*black*) and presence of UHRF1 (*green*) or SRA (*red*). *C*, stimulation of the activity of DNMT1 by UHRF1 and SRA (*green*; taken from Fig. 2*F*) and loss of the stimulation with DNMT1 variants (*blue* and *red*; average of three experiments). *Error bars* represent SEM.

In addition, we performed the DNMT1 stimulation assays using another setup and preincubated full-length UHRF1 or its SRA domain with the DNA substrate to form a protein-DNA complex. Afterward, the methylation reactions were started by the addition of DNMT1 and labeled AdoMet. These experiments showed no stimulation and even a slight inhibition of DNMT1 activity by both UHRF1 and the SRA domain (Fig. 3, *D* and *E*), which is in agreement with a finding published previously (43). This result can be explained by a competition of both proteins for the short oligonucleotide substrate used in our experiments. In summary, these data show that DNA binding of UHRF1 does not contribute to the stimulation of DNMT1 activity in our experiments. Therefore, the activation of DNMT1 must rely on the direct interaction between UHRF1 (via its SRA domain) and DNMT1 that leads to an allosteric activation of DNMT1. Our observation that the stimulation of DNMT1 depends on its preincubation with UHRF1 indicates that UHRF1-DNMT1 complexes form slowly but are stable in the time scale of our kinetics.

UHRF1 Stimulates the Activity of DNMT1 Mainly via an Interaction with the RFTS Domain—To understand the mechanism of the stimulation of DNMT1 by UHRF1, we aimed to identify the DNMT1 region involved in the interaction with UHRF1. Previous studies have mapped two regions of DNMT1 that interact with UHRF1: residues 401–615 corresponding to the RFTS domain (22, 26) and residues 1081–1408, which partially cover the BAH2 domain and the adjacent part of the catalytic domain (26). In our experiments, we used full-length DNMT1 and two truncated versions, amino acids 350–609 (the isolated RFTS domain) and 731–1602 (containing only the BAH1, BAH2, and catalytic domains) (11). The interaction between DNMT1 variants and the UHRF1 SRA domain was investigated using the Alpha Screen assay, which is a powerful system for analysis of protein-protein interactions because it is homophasic and requires only small amounts of protein. His-tagged DNMT1 proteins were immobilized on acceptor beads, and the GST-tagged SRA domain was immobilized on donor beads. The incubation of DNMT1 and GST was used as a control to determine the background of the assay (Fig. 4*A*). Nonspecific interactions were blocked by the presence of a 20-fold excess of BSA in the buffer. A strong Alpha signal was observed after incubation of full-length DNMT1 with the SRA domain. However, the highest Alpha signal was observed with the isolated RFTS domain and the SRA domain, indicating a strong interaction between these domains. Only a small Alpha signal was obtained after incubation of DNMT1(731–1602) and the SRA domain, indicating that the interaction between the SRA domain and the C-terminal part of DNMT1 is weak at best. Hence, the N-terminal part of DNMT1 containing the RFTS domain is needed for a strong interaction of DNMT1 with

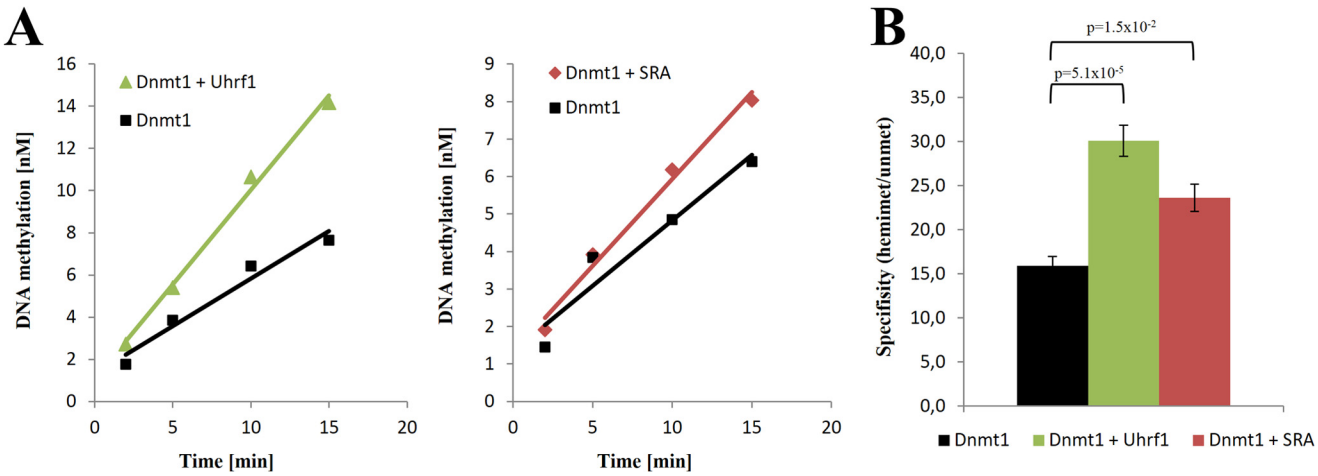

FIGURE 5. **UHRF1 increases the specificity of DNMT1.** *A*, example kinetics of the methylation of unmethylated DNA by DNMT1 in the absence (*black*) and presence of UHRF1 (*green*) or SRA (*red*) showing weaker stimulation of DNMT1 when compared with hemimethylated substrates (see Fig. 2*F*). *B*, specificity of DNMT1 expressed as the ratio of the rate of methylation of the hemimethylated substrate divided by the rate of methylation of unmethylated substrate. Specificity of DNMT1 was increased by UHRF1 and SRA from 16- to 30- and 24-fold, respectively (average of three experiments). *Error bars* represent SEM.

UHRF1, and this domain forms a strong contact to the SRA domain.

Based on these data, we speculated that the removal of the RFTS domain from DNMT1 might abolish the stimulatory effect of UHRF1 on DNMT1. To confirm this, we determined the DNA methylation activity of the DNMT1(731–1602) variant lacking the RFTS domain after incubation with UHRF1 or the SRA domain (Fig. 4, *B* and *C*). Neither SRA nor UHRF1 stimulated the activity of DNMT1(731–1602), which proves that the N-terminal part of DNMT1 is needed for UHRF1 and SRA to exert stimulation on DNMT1. To further confirm that UHRF1 interacts with the RFTS domain of DNMT1, we prepared a DNMT1 variant with mutations in the RFTS domain and replaced Glu-406 and Asp-407 (located on the upper surface of the domain) by two arginines. The DNMT1 E406R/D407R variant was catalytically active (even more than the wild type DNMT1), but it did not interact with the SRA domain in the Alpha Screen assay (Fig. 4*A*). We could not observe any stimulatory effect of the SRA domain and only observed a weak increase of the DNA methylation of this mutant in the presence of the full-length UHRF1 (Fig. 4, *B* and *C*). This result indicates that the RFTS domain mediates the interaction of DNMT1 and UHRF1, and this interaction is essential for the stimulation of DNMT1.

UHRF1 Improves the Specificity of DNMT1—Finally, we investigated whether UHRF1 influences the specificity of DNMT1. To this end, DNA methylation reactions were conducted with two 30-mer DNA substrates with an identical nucleotide sequence that differed only at the CpG site, which was hemimethylated in one substrate and unmethylated in the other. Both reactions were carried out either after preincubation of DNMT1 with UHRF1 or SRA or with buffer in the control samples. Under our experimental conditions, we observed an ~16-fold preference of DNMT1 for methylation of the hemimethylated over unmethylated DNA substrate, which is similar to previous observations by us and others (11, 13, 14, 49). We observed that the methylation of the unmethylated substrate is stimulated by UHRF1 and SRA only about 2.1- and 1.2-fold, respectively (Fig. 5*A*), which is around 2-fold less than the stimulation observed with the hemimethylated substrate (3.9- and 1.9-fold for UHRF1 and SRA, respectively). Hence, the interaction of DNMT1 with UHRF1 roughly doubles the preference of the methyltransferase for hemimethylated target sites (Fig. 5*B*).

DISCUSSION

The preferential substrate for DNMT1 is a hemimethylated CpG site, but the enzyme can also methylate unmethylated CpGs, and its specificity shown *in vitro* is not sufficient to ensure accurate maintenance of DNA methylation patterns. Additionally, on one hand, high activity of DNMT1 is required to ensure rapid remethylation of DNA after replication, whereas on the other hand, the activity of DNMT1 toward unmethylated DNA could lead to an aberrant DNA methylation, in particular during non-S phase, when no *bona fide* hemimethylated substrate is present. Hence, additional regulation of DNMT1 appears to be needed to fine-tune its activity and increase its specificity. UHRF1 is an essential factor in the maintenance of DNA methylation that increases the specificity of the methylation process by recruiting DNMT1 to newly replicated DNA at replication foci (26, 27).

UHRF1 Stimulates the Activity and Specificity of DNMT1—We showed here that UHRF1 further contributes to the DNA methylation maintenance process beyond targeting of DNMT1 because the interaction of DNMT1 with UHRF1 leads to an allosteric stimulation of the methyltransferase. Using *in vitro* DNA methylation assays, we demonstrated that DNMT1 preincubated with UHRF1 methylates hemimethylated DNA up to 5 times faster than in control reactions. Also, we observed a 2-fold increase in the specificity of DNMT1 toward the hemimethylated DNA in the presence of UHRF1, which suggests that the interaction of UHRF1 with DNMT1 also increases the recognition of hemimethylated DNA. This observation demonstrates that UHRF1 acts as a multifunctional regulator of DNMT1 because it targets the enzyme to hemimethylated DNA and additionally increases its activity and specificity.

UHRF1 Stimulates DNMT1 in an Allosteric Manner—We showed that UHRF1 stimulates the activity of DNMT1 in a

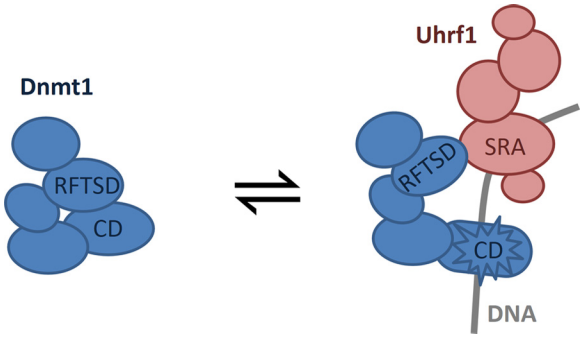

FIGURE 6. **Model of the recruitment and allosteric activation of DNMT1 by UHRF1.** In DNMT1 (*blue*), the RFTS domain prevents access to the catalytic domain (*CD*). UHRF1 (*red*) binding to the RFTS domain leads to unblocking of the catalytic domain of DNMT1. DNA containing hemimethylated CpG sites can bind to the SRA domain of UHRF1 and the catalytic domain of DNMT1.

DNA binding-independent manner. Thus, the interaction of UHRF1 with DNA is important for targeting of DNMT1 to the hemimethylated DNA but not required for the allosteric activation of DNMT1 by UHRF1. In contrast to our results, Felle *et al.* (43) did not observe an increase in the activity of DNMT1 in the presence of UHRF1. This can be explained by the experimental setup used by Felle *et al.* (43) because they preincubated UHRF1 with DNA and then started the reaction by adding DNMT1 and AdoMet. Using the same setup, we obtained similar results, suggesting that UHRF1 competes with DNMT1 for the short oligonucleotide substrate in the *in vitro* reactions and thereby decreases DNA methylation.

Our results make a model in which UHRF1 binds to a hemimethylated CpG site via its SRA domain and then recruits DNMT1 directly to this CpG site unlikely (23). A direct transfer of a CpG site from the SRA domain to DNMT1 is not in agreement with our data because preincubation of the SRA domain with the oligonucleotide substrates containing one hemimethylated CpG site inhibited DNMT1 activity, indicating that a direct "handing over" does not occur.

Mechanism of Regulation of DNMT1 by UHRF1—We mapped the interaction between DNMT1 and UHRF1 to the RFTS domain of DNMT1 that has been shown to be involved in directing DNMT1 to replication foci (50). Recently, it was shown by crystallography that in the absence of DNA the RFTS domain occupies the DNA binding pocket in the catalytic domain of DNMT1 and behaves as an autoinhibitor for DNMT1 (19, 20). For active DNA methylation, DNMT1 needs to adopt a different conformation in which the RFTS domain is released from the catalytic domain such that the enzyme can bind and methylate DNA. Our data suggest that free DNMT1 is in a weakly active conformation that reduces undesired *de novo* methylation. Binding of DNMT1 to UHRF1 induces a transition of the enzyme into a more active conformation and stimulates the activity of DNMT1 by inducing the release of the RFTS domain from the catalytic domain. In addition, UHRF1 enhances the specificity of DNMT1 and thus improves the accuracy of the DNA methylation maintenance (Fig. 6). In cells, UHRF1 may either bind to hemimethylated DNA already in complex with DNMT1 or it may bind in free form and then recruit DNMT1. Afterward, DNMT1 can bind to the DNA at a site close to the UHRF1 binding site. This targeting process and the simultaneous allosteric activation of DNMT1 by UHRF1 efficiently direct DNA methylation to a DNA region containing hemimethylated CpG sites. The physiological importance of this process is illustrated by the fact that UHRF1 is essential for maintenance of DNA methylation in cells (23, 26).

It is interesting to compare the role and mechanism of UHRF1 with those of other regulators of DNMTs. DNMT3L is a regulator of DNMT3A needed for the setting of parental imprints in germ cells. Like UHRF1, DNMT3L stimulates the activity of DNMT3A (51–53), and it also changes its localization (54), suggesting that concomitant targeting and stimulation of DNMTs are a general approach in the regulation of this class of enzymes.

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Addendum—After submission of this article, a study by Berkyurek et al. (55) appeared in press, reporting the stimulation of a DNMT1(291–1620) fragment by the UHRF1 SRA domain, which is in agreement with our results.

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The UHRF1 Protein Stimulates the Activity and Specificity of the Maintenance DNA Methyltransferase DNMT1 by an Allosteric Mechanism

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